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(54) Title: PEAR GENES CODIFYING FOR β-GALACTOSIDASE, PECTIN METHYLESTERASE, POLYGALACTURONASE, EXPANSINS AND THEIR USE

(57) Abstract: This invention provides isolated and purified nucleotide sequences which are differentially expressed during pear fruit ripening, and their protein products. The isolated genes can be inserted into expression cassettes and cloned in an expression vector which can be used to transform a host cell by selected transformation methods. Transgenic plants can be regenerated from transformed plantss cells by in vitro culture techniques. The nucleotide sequences disclosed in this invention encode proteins which are described as having an effective action in fruit ripening control. When used in antisense orientation they can delay fruit softening and mesocarp deterioration, bringing important advantages for fruit producers.

DESCRIPTION

Pear genes codifying for β-Galactosidase, Pectin Methylesterase, Polygalacturonase, Expansins and their use.

FIELD OF THE INVENTION

The present invention relates to the isolation and identification of nucleotide sequences encoding for proteins involved in ripening pear fruits, a method for regulating fruit ripening by transforming plants with a construct containing one or more of the isolated genes, and transgenic plants and seeds transformed with such constructs.

BACKGROUND OF THE INVENTION

Pears are the third most important fruit produced in temperate regions after grapes and apples.

Pear (*Pyrus communis* L.) epidermis is very sensitive to transport and handling, small mechanical shocks give rise to mesocarp deterioration and precocious pear senescence. Pears are harvested at commercial maturity (a full growing green stage) and cold stored. The onset of ripening starts when the fruits leave the cold, and it takes only two weeks until the fruit reaches an overripe phase. This means that most of the time when pear fruits reach the consumers they are overripen. To avoid this, the producers have to harvest pears before they reach the optimal maturation stage. Often these fruits fail to ripen with full organoleptic quality. This constitutes a problem for fruit producers, which has considerable losses in fruit flowing off, and for consumer, which often buy a fruit with poor quality. For all that we can understand why only about 10% of the pears produced in Portugal, for example, are exported (Azevedo, 1997, Revista do Agricultor 104/105:45-48).

At the present time producers have the need to control pear fruit ripening so they started to test the application of chemical products to delay fruit ripening. The molecular approach described in this patent provides the ripening control by antisense expression of ripening related genes without use of chemical substances and with no changes in the organoleptic characteristics of such tasty fruit.

Extensive cell wall modifications that occur during fruit ripening are thought to underlie processes such as fruit softening, tissue deterioration, and pathogen susceptibility. These modifications are regulated at least in part by the expression of genes that encode cell wall-modifying enzymes (Fisher and Bennett, 1991, Annu. Rev. Plant Physiol. Plant Mol. Biol., 42:675-703). Pectins are a major class of cell wall polysaccharides that are degraded during ripening, undergoing both solubilization and depolymerization. In tomato the majority of ripening-associated pectin degradation is attributable to the cell wall hydrolase Polygalacturonase (Hadfield et al., 1998, Plant Physiol., 117:363-373).

Polygalacturonase (PG) catalyze the hydrolytic cleavage of α-(1$\rightarrow$4) galacturonan linkages of pectic backbone (Fisher and Bennett, 1991, Annu. Rev. Plant Physiol. Plant Mol. Biol., 42:675-703). PG has been extensively studied in tomato fruit, where it accumulates during ripening and is responsible for the degradation of polyuronides in fruit cell wall (Smith et al., 1988, Nature, 334:724-726). However, experiments using transgenic tomato plants with altered PG gene expression indicated that PG-dependent pectin degradation is neither required nor sufficient for tomato fruit softening to occur (Sheehy et al., 1988, Proc. Natl. Acad. Sci. USA, 85:8805-8809; Smith et al., 1988, Nature, 334:724-726; Giovannonni et al., 1989, Plant Cell, 1:53-63). Data from experiments using fruit of the same transgenic lines strongly suggested that PG-mediated pectin degradation is important in the later, deteriorative stages of ripening and in pathogen susceptibility of tomato fruit (Schuch et al., 1991, Hortscience, 26-:1517-1520; Kramer et al., 1992, Post. Biol. Tech., 1:241-255; Hadfield et al., 1998, Plant Physiol., 117:363-373).

Polygalacturonase is known to be more active in degrading demethylated than methylated pectin (Fisher and Bennett, 1991, Annu. Rev. Plant Physiol. Plant Mol. Biol., 42:675-703). Pectin methylesterase (PME) is a cell wall metabolizing enzyme responsible for the demethylation/de-esterification of galacturonic acid residues in high molecular weight pectin (Hall et al., 1993, The Plant J., 3(1): 121-129). In tomato, PME is present throughout fruit development with activity increasing two to three-fold during ripening (Hobson, 1963, Biochem. J., 86:358-365; Harriman et al., 1991, Plant Physiol., 97:80-87). As the methylesterification level (60%) seems to protect the homogalacturonans (HGA) from a more extended PG activity, it has been

thought that PME play an important role in the determination of the extension in which the pectins are susceptible to PG action (Dick and Labavitch, 1989, Plant Physiol., 89:1394-1400). Inhibition of fruit-specific PME gene expression by its antisense gene, in tomato, results in loss of tissue integrity of fruit pericarp but does not affect the growth and development of tomato plant (Tieman et al., 1992, Plant Cell, 4:667-679; Hall et al., 1993, The Plant J. 3(1): 121-129; Tieman and Handa, 1994, Plant Physiol., 106:429-436).

Although some loss of galactosyl residues could result indirectly from the action of PG, β-Galactosidase (β-Gal) is the only enzyme identified in higher plants capable of directly cleaving β-(1,4) galactan bonds, and probably plays a role in galactan side chain loss (De Veau et al., 1993, Physiol. Plantarum, 87:279-285; Carey et al., 1995, Plant Physiol., 108:1099-1107; Carrington and Pressey, 1996, J. Am. Soc. Hortic. Sci., 121:132-136; Smith et al., 1998, Plant Physiol., 117:417-423). Studies in apple, melon, kiwi and avocado (Ranwala et al., 1992, Plant Physiol., 100:1318-1325; Ross et al., 1993, Planta, 189:499-506; Ross et al., 1994, Plant Physiol., 106:521-528) suggests that β-Gal acts like a galactanase hidrolyzing the neutral sugar polimers which attach the ramnogalacturonan backbone from pectins to the hemicelluloses (Lazan et al., 1995, Physiol. Plantarum, 95:106-112; Ranwala et al., 1992, Plant Physiol., 100:1318-1325). Several studies suggest that β-gal can significantly contribute to pectin and hemicellulose modification, assuming an especially important role in the later stages of fruit ripening. That activity could be complemented by PG, cellulases and other glycosidases action (Carey et al., 1995, Plant Physiol., 108:1099-1107).

Unlike the enzymes described above, Expansins lack hydrolytic activity (McQueen-Mason et al., 1992, Plant Cell, 4:1425-1433; McQueen-Mason et al., 1993, Planta, 190:327-331). Instead, Expansins appear to disrupt the noncovalent bonding between cellulose and hemicellulose, thereby allowing the wall polymers to yield to the turgor-generated stresses in the cell wall (Cosgrove, 1997, Proc. Natl. Acad. Sci. USA, 94:5504-5505). This results in a relaxation of wall stress and turgor pressure and, consequently, an uptake of water to enlarge the cell and expand the wall (Cosgrove, 1993, New Phytol., 124:1-23; Scherban et al., 1995, Proc. Natl. Acad. Sci. USA. 92:9245-9249). Expansin protein motifs are very conserved, however they play

a role in different processes of cellular growth. An expansin gene from tomato was recently isolated and showed to be specifically and abundantly expressed in ripening fruit, when growth ceased and a strong cell wall degradation occurs (Rose et al., 1997, Proc. Natl. Acad. Sci. USA, 94:5955-5960; Rose et al., 2000, Plant Physiol., 123:1583-1592). Homolog cDNAs have already been isolated from other rapid ripening fruits like melon and strawberry. It is known that expansin expression is ethylene regulated which makes us to assume these proteins can also contribute to cell wall degradation in non-growing tissues, allowing a more efficient action of other endogenous enzymes on non-covalently linked polymers (Rose et al., 1997, Proc. Natl. Acad. Sci. USA, 94:5955-5960).

SUMMARY OF THE INVENTION

Genes codifying for β-Galactosidase, Pectin Methylesterase, Polygalacturonase and two Expansin proteins were isolated from pear fruit. These enzymes are expressed during fruit maturation and ripening and can be used as targets for the generation of transgenic plants. The isolated genes can regulate the referred enzyme expression and thereby control aspects of plant development, and in particular fruit ripening.

These genes can be inserted in sense or antisense in pear and in other fruit species allowing the ripening control. By "antisense downregulation" and "sense downregulation or "cossupression", the expression of a target gene can be inhibited. As a consequence the fruits can be collected later on ripening, with better organoleptic quality and reduced losses in transportation and storage.

DETAILED DESCRIPTION AND PREFERED EMBODIMENTS OF THE INVENTION

The present invention provides new isolated genes from pear fruit particularly produced during the ripening process. These genes encode for cell wall hydrolases - β-Galactosidase (β-Gal), Pectin Methylesterase (PME) and Polygalacturonase (PG) - and for a novel class of cell wall proteins - Expansins (Exp1 and Exp2).

Also provided for this invention, the claimed nucleic acid sequence can be used to suppress the expression of endogenous β-gal, PME, PG, Exp1, and Exp2 genes in any fruit or other plant organs, thus modifying the structure of the cell walls of the fruit or

plant and providing for ripe yet firm fruit and vegetables. This suppression can be achieved by "sense downregulation" or "cossuppression" or by "antisense downregulation". mRNA, RNA, cRNA, cDNA and DNA molecules inserted in sense or antisense orientation can serve this purpose.

Nucleic Acids Sequences Isolation from Plants

The genes of the present invention may be isolated from ripening fruits using different methods well known in the art. In particular two approaches can be used. One is the approach described here which consists on degenerated primers design from conserved portions of sequence alignments, using sequences from the same gene isolated from other species published in the database. The other approach can be the construction of a cDNA library and screening using heterologous probes.

The procedures for isolating the DNA, RNA or cDNA encoding a protein according to the present invention, subjecting it to partial digestion, isolating DNA fragments, ligating the fragments into a cloning vector, and transforming a host are well known in recombinant DNA technology. Accordingly, one of ordinary skill in the art can use or adapt the detailed protocols for such procedures as found in Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual, 2nd. Ed., Cold Spring Harbor, or any other manual on recombinant DNA technology. Fragments of the genes of the present invention are also contempled by the present invention.

The designed degenerated primers can be used to obtain isoenzymes of the same gene in Pyrus species or to isolate the homologous gene from other different species by PCR and other in vitro amplification methods. The specific designed primers can be replaced by different ones in order to obtain slightly different fragments of the same nucleic acid sequence claimed here. For a general overview of PCR see PCR Protocols: A Guide to Methods and Applications (Innis, M., Gelfand, D., Sninsky, J., and White, T., eds.) Academic press, San Diego (1990).

Polynucleotides can also be synthesized by well-known techniques as described in the technical literature. Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Once one coding gene of the present invention has been isolated from species, it can serve as a hybridization probe to isolate corresponding genes from the other species by cross-hybridization under low or moderate stringency conditions. Used as heterologous probes, the isolated genes can be used for screening a cDNA library or a genomic library, from any species. Used as homologous probes, the isolated nucleic acid sequences can be used to screen a library constructed from any species of Pyrus genus.

Substitution of one or more codons coding for an amino acid having similar chemical properties to the original one can be made creating an analog-coding gene. An analog may be defined as a peptide or fragment which exhibits the biological activity of the proteins of the present invention, and which is differentially expressed during fruit ripening.

Use of Nucleic Acids of the Invention to Inhibit Gene Expression

According to the present invention, a DNA molecule may also be operably linked to a promoter capable of regulating the expression of the said DNA molecule, to form a chimeric gene. That chimeric gene can be introduced into a replicable expression vector, for using in transforming plants. The replicable expression vectors may also be used to obtain the polypeptides coded by the genes of the present invention by well-known methods in recombinant DNA technology.[S1]

Replicable expression vectors usually comprise a promoter (at least), a transcription enhancer fragment, a termination signal, a translation signal, or a combination of two or more of these elements operably linked in proper reading frame. Preferably the vector encodes also a selectable marker, for example, antibiotic resistance. Replicable expression vectors can be plasmids, cosmids, bacteriophages and viruses.

The isolated sequences can be used to prepare expression cassettes useful in a number of techniques. For example, these expression cassettes can be used to suppress endogenous Exp1 or Exp2 gene expression. Inhibiting expression can be useful, for instance, in suppressing the extension of plant cell walls and disassembly of cell wall components.

The nucleic acid segment to be introduced generally will be substantially identical to at least a portion of the endogenous gene or genes to be repressed. However the sequence does not need to be perfectly identical to inhibit expression.

Several methods can be used to inhibit gene expression in plants, using the antisense technology. A nucleic acid segment of the interest gene can be operably linked to a promoter (CaMV 35S promoter or to a fruit specific promoter, for example) such that the antisense strand of RNA will be transcribed. That expression cassette can be then used to transform plants were the antisense strand of RNA will be produced. In plant cells, it has been suggested that antisense RNA inhibits gene expression by preventing the accumulation of mRNA which encodes the enzyme of interest, see e.g., van der Krol et al., 1988, Gene, 72:45-50.

For antisense supression generally higher homology can be used to compensate for the use of a shorter sequence. Normally, a sequence about 30 or 40 nucleotides and about full-length nucleotides can be used, but sequences between 200 and 500 nucleotides are especially preferred.

Catalytic RNA molecules or ribozymes can also be used to inhibit expression of the claimed genes. It is possible to design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. The inclusion of rybozime sequences within antisense RNAs confers RNA activity upon them, thereby increasing the activity of the constructs.

Another method of suppression is sense suppression. Introduction of expression cassettes in which a nucleic acid or a nucleic acid fragment is positioned in the sense orientation in frame with the promoter has shown to be an effective mean to block the transcription of target endogenous genes. See as revision article Stam et al., 1997, Annals of Botany, 79:3-12.

When sense inhibition of expression is desired, the introduced sequence should contain at least a fragment of the coding sequence or an intron or untranslated sequences homologous to sequences present in the primary transcript of the endogenous sequence. The introduced sequence should be substantially identical to the endogenous sequence intended to be repressed. The minimal identity should be

typically greater than about 65%, but identities comprised between 80 to 100% are preferred. As in antisense suppression a higher identity in a shorter than full-length sequence compensates for a longer, less identical sequence. Nucleic acid sequences about 30 or 40 nucleotides may be used, but sequences between 200 and 500 nucleotides are especially preferred.

Use of Nucleic Acids of the Invention to Enhance Gene Expression

In opposition to the inhibiting fruit softening process, the nucleotide sequences of the invention can be used to accelerate the cell wall disassembly. This can be accomplished by the overexpression of the isolated sequences.

Use of Nucleic Acids of the Invention to Produce Transgenic Plants

The nucleic acid sequences isolated in the present invention can be incorporated in an expression vector and thereby be introduced into a host cell. Accordingly, one skilled in the art can use the sequences to make a recombinant cell. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like. Preferably the host cells are either a bacterial cell or a plant cell.

The nucleotide sequences claimed in this invention can be inserted in an expression vector, which may be introduced into the genome of the desired plant host by a variety of conventional techniques. The constructions using the isolated genes can be introduced into a conventional Agrobacterium tumefaciens host vector. The virulence functions of the Agrobacterium host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the bacteria infect the cell.

Alternatively, the DNA constructs can be directly introduced into the plant cell genomic DNA using techniques such as electroporation and microinjection in plant cell protoplasts. Ballistics methods, such as DNA particle bombardment allows the DNA to be introduced directly in plant tissue.

Transformed plant cells derived by any of the above transformation techniques can be cultured to generate a whole plant, which possesses the transformed genotype and thus the desired phenotype such as increased fruit firmness. Such regeneration techniques rely on the manipulation of certain nutrients and phytohormones in a culture medium containing an antibiotic, herbicide or other marker that has been

introduced together with the nucleotide sequences of interest. Regeneration can also be obtained from different plant explants or embryos. For a general overview, see Plant Cell, Tissue and Organ Culture. Fundamental Methods (O.L. Gamborg and G.C. Philips, eds.) Springer-Verlag (1995). Plant tissues suitable for transformation include, but are not limited to, floral buds, leaf tissue, root tissue, meristems, zygotic and somatic embryos, anthers, microspores and megaspores.

The resulting transformed plant with the genes of this invention may have an over expression or silencing pattern of β-gal and/or PME and/or PG and/or Exp1 and/or Exp2 genes. These plant fruits may have an abnormal ripening behavior: slower pulp softening, later mesocarp deterioration, increased fruit shelf life after harvest and an enhanced resistance against pathogenic attack. That is an example, if the isolated nucleotide sequences were used aiming the corresponding enzyme downregulation.

Fruit ripening control can be achieved in the transformed plants with constructions containing the isolated cDNA sequences. Moreover, the alterations produced in fruit tissue at cell wall level can interfere with the response to pathogens attack, namely to fungal attack, delaying or decreasing the extension of pathogen infection.

The DNA molecules of the present invention may be used to transform any plant in which expression of the particular protein encoded by said DNA molecules is desired. The DNA molecules of the present invention can be used over a broad range of plants, namely species from genera such as Asparagus, Avena, Brassica, Citrus, Citrullus, Capsicum, Castanea, Cucurbita, Daucus, Fragaria, Glycine, Hordeum, Lactuca, Licopersicon, Malus, Manihot, Nicotiana, Oryza, Persea, Pisum, Pyrus, Prunus, Raphanus, Secale, Solanum, Sorghum, Triticum, Vitis, Vigna, and Zea. The β-gal, PME, PG, Exp1 and Exp2 genes are particularly useful in the production of transgenic plants of Pyrus genus. It has to be understood that is not an exclusive list, but merely suggestive of the wide range of applicability of the DNA molecules of the present invention.

Any skilled person will recognize that an enzymatic activity assay, immunoassay, western blotting and other detection assays can be used to detect at the protein level, the presence or absence of the proteins which the isolated sequences encode for. At DNA level, Southern blotting, northern blotting and PCR analyses can be performed

in order to determine, the effective integration of the desired gene sequences in the plant DNA, and the efficient gene expression or silencing due to the introduced sequences.

Any skilled person will recognize that after an expression cassette being stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. A number of standard breeding techniques can be used, depending on the species to be crossed. Transgenic seeds and propagules (e.g., cuttings) can be obtained and when cultured produce transgenic plants.

The embodiments described above and the following examples are provided to better illustrate the practice of the present invention and should not be used to limit the scope of the invention. It is understood that the invention is not restricted to the particular material, combinations of material, and procedures selected for that purpose. Numerous variations of such details can be implied and will be appreciated by those skilled in the art.

EXAMPLES

Example1

Amplification of a β-galactosidase gene from pear (Pcβgal)

Rocha Pear (*Pyrus communis* L. cv. Rocha) fruit mesocarp at different maturation stages was frozen in liquid nitrogen, grounded to a fine powder in a mortar and stored at -80 °C. About 6 g of powder were mix with 20 ml of RNA extraction buffer for RNA extraction according the hot borate protocol (Wan and Wilkins, 1994, Anal. Biochem., 223:7-12). Messenger RNA (mRNA) isolation was performed with the Poly A Ttract System (Promega) according to manufacturer instructions. The RNA and mRNA pellet was stored in DEPC treated water at -80°C. Spectrophotometric quantification was performed in TE buffer. RNA and mRNA were electrophoresed on a 0.8 % agarose gel at 80 V for 1.5 hr to check its integrity.

For the reverse transcription reaction (RT), 1 μg of pear mRNA and 25 U of Avian Myeloblastosis Virus (AMV) reverse transcriptase in a reaction mixture of 50 mM Tris-HCl pH 8.5, 8 mM MgCL2, 30 mM KCl and 1 mM DTT, containing 1.0 mM

each dNTP, 12.5 μg BSA, 1.25 μg actinomicin D and 10 μM of oligo (dT) 17 (provided with 5'/3'Race kit, Boehringer) was incubated for 90 min at 55°C. The cDNA produced was amplified with 2.0 U Taq DNA polymerase (Gibco-BRL) in a 20 mM Tris-HCl pH 8.4 and 50 mM KCl mixture containing 2.0 mM MgCl2, 0.25 mM each dNTP and 10 pmol of each degenerated primers BG1 (SEQ. ID. NO: 17) and BG2 (SEQ. ID. NO: 18). After an initial 5 min denaturation period at 94 °C, the PCR parameters were 30 sec template denaturation at 94 °C, 45 sec primer annealing at 45 °C and 2 min primer extension at 72 °C for 35 cycles. A final extension step of 10 min at 72 °C was used subsequently to ensure full-length amplification products. The termocycler used was a Perkin Elmer - Gene Amp PCR System 2400.

The obtained products were purified from the agarose gel and ligated into the vector pBluescript (KS+) (Stratagene). The ligated mixture was used to transform E. coli DH5α. Transformants were selected on LB agar plates containing ampicilin (100 μg/ml) X-gal (80 μg/ml) and IPTG (0.5 mM). Plasmid DNA was isolated using alkaline lysis method.

DNA sequencing was performed in an automated sequencer ABI 310 Applied Biosystems, using Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

The two bands obtained by PCR have approximately 2.0 and 2.3 Kb. The nucleotide sequences were sent to NCBI data bank that has shown significant homology with β-galactosidases isolated from other species. Both obtained bands correspond to the same gene sequence resulting, the smaller one from amplification with BG1 (SEQ. ID. NO: 17) and BG2 (SEQ. ID. NO: 18) primers, and the larger one from BG1 (SEQ. ID. NO: 17) and oligo (dT) 17 primer (Boehringer) (which has been used in the RT reaction). As the obtained sequence corresponds to about 90% of the gene coding region, a new specific antisense primer BG3 (SEQ. ID. NO: 19) (see Table 1) was designed to perform 5' RACE (Rapid Amplification of cDNA Ends) reaction.

In order to perform 5' RACE reactions, Marathon kit (Clontech) cDNA synthesis reaction was done using 4 μg of pear mRNA. The adapter ligation allows the use of AP1 (Adaptor Primer, provided with Marathon kit, Clontech) primer in amplification reaction. Marathon cDNA was amplified with 2.0 U Taq DNA polymerase (Gibco-

BRL) in a 20 mM Tris-HCl pH 8.4 and 50 mM KCl mixture containing 2.0 mM MgCl2, 0.25 mM each dNTP and 10 pmol of primers BG3 (SEQ. ID. NO: 19) (see Table1) and AP1 (Clontech). After an initial 5 min denaturation period at 94 °C, the PCR parameters were 30 sec at 94 °C, 45 sec at 60 °C and 45 sec at 72 °C for 35 cycles and a final extension step of 10 min at 72 °C. The 150 bp PCR product was cloned and sequenced as described above.

Fused together the 2.3 Kb sequence and the 0.150 Kb sequence represented about 95% of the complete coding region for pear β-galactosidase protein.

The β-galactosidase nucleotide sequences (SEQ. ID. NO:1) was sent to NCBI data bank and has shown significant homology with β-galactosidases isolated from other species. The highest homology found at the DNA level using the blastn program was 96% with Pyrus pyrifolia mRNA clone # AB046543. Searches in all the available protein and DNA data banks failed to find 100 % homology with any existing clone.

Example 2

Amplification of a Polygalacturonase gene from pear (PcPG)

Pear mesocarp processing, RNA extraction, mRNA isolation and RT reaction were performed exactly as described for β-galactosidase isolation in Example 1.

The cDNA produced was amplified with 2.0 U Taq DNA polymerase (Gibco-BRL) in a 20 mM Tris-HCl pH 8.4 and 50 mM KCl mixture containing 2.0 mM MgCl2, 0.25 mM each dNTP and 10 pmol of each degenerated primers PG1 (SEQ. ID. NO:20) and PG2 (SEQ. ID. NO:21) (see Table1). After an initial 5 min denaturation period at 94 °C, the PCR parameters were 30 sec template denaturation at 94 °C, 30 sec primer annealing at 55 °C and 45 sec primer extension at 72 °C for 35 cycles. A final extension step of 10 min at 72 °C was used subsequently to ensure full-length amplification products. The termocycler used was a Perkin Elmer - Gene Amp PCR System 2400.

The obtained product was purified from the agarose gel and ligated into the vector pBluescript (KS+) (Stratagene). The ligated mixture was used to transform E. coli

DH5α. Transformants were selected on LB-agar plates containing ampicilin (100 μg/ml) X-gal (80 μg/ml) and IPTG (0.5 mM). Plasmid DNA was isolated using alkaline lysis method.

DNA sequencing was performed in an automated sequencer ABI 310 Applied Biosystems, using Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

The PCR obtained band has approximately 160 bp that corresponds only to 10 % of coding region. In order to isolate whole gene RACE reactions were performed - 5' RACE reaction using the Marathon cDNA and 3' RACE using cDNA from an RT performed as described in Example 1. Also, new primers were designed: PG3 (an antisense primer for 5' RACE) (SEQ. ID. NO:22) and PG4 (a sense primer for 3' RACE) (SEQ. ID. NO:23).

For 5' RACE reaction, Marathon cDNA was amplified with 2.0 U Taq DNA polymerase (Gibco-BRL) in a 20 mM Tris-HCl pH 8.4 and 50 mM KCl mixture containing 2.0 mM MgCl2, 0.25 mM each dNTP and 10 pmol of primers PG3 (SEQ. ID. NO:22) (see Table 1) and AP1 (provided with Marathon kit, Clontech). After an initial 5 min denaturation period at 94 °C, the PCR parameters were 30 sec at 94 °C, 45 sec at 52 °C and 1 min 20 sec at 72 °C for 35 cycles and a final extension step of 10 min at 72 °C. The approximately 700 bp PCR product was cloned and sequenced as described above.

For the 3' RACE reaction cDNA was amplified with 2.0 U Taq DNA polymerase (Gibco-BRL) in a 20 mM Tris-HCl pH 8.4 and 50 mM KCl mixture containing 2.0 mM MgCl2, 0.25 mM each dNTP and 10 pmol of primers PG4 (SEQ. ID. NO:23) (see Table1) and Vial9 primer (provided with 5'/3' Race kit, Boehringer). After an initial 5 min denaturation period at 94 °C, the PCR parameters were 30 sec at 94 °C, 45 sec at 45 °C and 2 min at 72 °C for 35 cycles and a final extension step of 10 min at 72 °C. The approximately 800 bp PCR product was cloned and sequenced as described for the 160 bp fragment.

All the three isolated polygalacturonase fragments together comprise a cDNA molecule of 1673 bp in size (SEQ. ID. NO:3) and represent 100 % of the coding region. The complete nucleotide sequence was sent to NCBI data bank and has shown

significant homology with polygalacturonases isolated from other species. The highest homology found at the DNA level using the blastn program was 81% with Prunus persica mRNA clone # AF095577. Searches in all the available protein and DNA data banks failed to find 100 % homology with any existing clone.

Example 3

Amplification of a Pectin Methylesterase gene from pear (PcPME)

Pear mesocarp processing, RNA extraction, mRNA isolation and RT reaction were performed exactly as described for β-galactosidase isolation in Example 1.

The cDNA produced was amplified with 2.0 U Taq DNA polymerase (Gibco-BRL) in a 20 mM Tris-HCl pH 8.4 and 50 mM KCl mixture containing 3.0 mM MgCl2, 0.25 mM each dNTP and 20 pmol of each primer PME1 (SEQ. ID. NO:24) and PME2 (SEQ. ID. NO:25) (see Table1). After an initial 5 min denaturation period at 94 °C, the PCR parameters were 30 sec template denaturation at 94 °C, 30 sec primer annealing at 50 °C and 1 min primer extension at 72 °C for 35 cycles. A final extension step of 10 min at 72 °C was used subsequently to ensure full-length amplification products. The termocycler used was a Perkin Elmer - Gene Amp PCR System 2400.

The obtained product was purified from the agarose gel and ligated into the vector pBluescript (KS+) (Stratagene). The ligated mixture was used to transform E. coli DH5α. Transformants were selected on LB agar plates containing ampicilin (100 μg/ml) X-gal (80 μg/ml) and IPTG (0.5 mM). Plasmid DNA was isolated using alkaline lysis method.

DNA sequencing was performed in an automated sequencer ABI 310 Applied Biosystems, using Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

The PCR obtained band has approximately 200 bp that corresponds only to 15 % of coding region. In order to try to isolate whole gene a 5'RACE reaction was performed using the Marathon cDNA. Also a new primer was designed: PME3 (an antisense primer for 5' RACE) (SEQ. ID. NO:26)

For 5' RACE reaction, Marathon cDNA was amplified with 2.0 U Taq DNA polymerase (Gibco-BRL) in a 20 mM Tris-HCl pH 8.4 and 50 mM KCl mixture containing 2.0 mM MgCl2, 0.25 mM each dNTP and 10 pmol of primers PME3 (SEQ. ID. NO:26) (see Table 1) and AP1 (provided with Marathon kit, Clontech). After an initial 5 min denaturation period at 94 °C, the PCR parameters were 30 sec at 94 °C, 30 sec at 50 °C and 1 min at 72 °C for 35 cycles and a final extension step of 10 min at 72 °C. The approximately 600 bp PCR product was cloned and sequenced as described above.

Both fragments together comprise a cDNA molecule of 700 bp in size (SEQ. ID. NO:5) and represents about 60 % of the coding region.

The PME nucleotide sequence was sent to NCBI data bank and has shown significant homology with pectin methylesterases isolated from other species. Searches in all the available protein and DNA data banks failed to find 100 % homology with any existing clone.

Example 4

Amplification of two Expansin genes from pear (PcExp1 and PcExp2)

Pear mesocarp processing, RNA extraction, mRNA isolation and RT reaction were performed exactly as described for β-galactosidase isolation in Example 1.

The cDNA produced was amplified with 2.0 U Taq DNA polymerase (Gibco-BRL) in a 20 mM Tris-HCl pH 8.4 and 50 mM KCl mixture containing 2.0 mM MgCl2, 0.25 mM each dNTP and 10 pmol of each degenerated primers EX1 (SEQ. ID. NO:27) and EX2 (SEQ. ID. NO:28) (see Table1). After an initial 5 min denaturation period at 94 °C, the PCR parameters were 30 sec template denaturation at 94 °C, 30 sec primer annealing at 58 °C and 45 sec primer extension at 72 °C for 35 cycles. A final extension step of 10 min at 72 °C was used subsequently to ensure full-length amplification products. The termocycler used was a Perkin Elmer - Gene Amp PCR System 2400.

An approximately 300 bp expected band was obtained. This product was purified from the agarose gel and ligated into the vector pBluescript (KS+) (Stratagene). The

ligated mixture was used to transform E. coli DH5α. Transformants were selected on LB agar plates containing ampicilin (100 μg/ml) X-gal (80 μg/ml) and IPTG (0.5 mM). Plasmid DNA was isolated using alkaline lysis method. DNA sequencing was performed in an automated sequencer ABI 310 Applied Biosystems, using Big Dye Terminator Cycle Sequencing kit (Applied Biosystems).

The PCR obtained band of approximately 300 bp corresponds only to 30 % of the coding region. In order to isolate whole gene RACE reactions were performed - 5' RACE reaction using the Marathon cDNA and 3' RACE using cDNA from an RT performed as described in Example 1. Also, new primers were designed: EX3 (SEQ. ID. NO:29) (an antisense primer for 5' RACE) and EX4 (SEQ. ID. NO:30) (a sense primer for 3' RACE).

For 5' RACE reaction, Marathon cDNA was amplified with 2.0 U Taq DNA polymerase (Gibco-BRL) in a 20 mM Tris-HCl pH 8.4 and 50 mM KCl mixture containing 2.0 mM MgCl2, 0.25 mM each dNTP and 10 pmol of EX3 (SEQ. ID. NO:29) (see Table 1) and AP1 (Adaptor Primer provided with Marathon kit, Clontech) primers. After an initial 5 min denaturation period at 94 °C, the PCR parameters were 30 sec at 94 °C, 45 sec at 42 °C and 1 min at 72 °C for 35 cycles and a final extension step of 10 min at 72 °C. When cloned, the approximately 500 bp PCR product showed two distinct patterns when cut with EcoRI and Hind III restriction enzymes. Both clones were then sequenced and revealed to be different expansin gene fragments. The first one corresponds to 5' region of the 300 bp Expansin 1 gene isolated. The second one was Expansin 2 5' end.

For the 3' RACE reaction of Exp1, cDNA was amplified with 2.0 U Taq DNA polymerase (Gibco-BRL) in a 20 mM Tris-HCl pH 8.4 and 50 mM KCl mixture containing 2.0 mM MgCl2, 0.25 mM each dNTP and 10 pmol of each EX4 (SEQ. ID. NO:30) (see Table1) and Vial9 primers (provided with 5'/3' Race kit, Boehringer). After an initial 5 min denaturation period at 94 °C, the PCR parameters were 30 sec at 94 °C, 45 sec at 48 °C and 1 min at 72 °C for 35 cycles and a final extension step of 10 min at 72 °C. The approximately 700 bp PCR product was cloned and sequenced.

For the 3' RACE reaction of Exp2, cDNA was amplified with 2.0 U Taq DNA polymerase (Gibco-BRL) in a 20 mM Tris-HCl pH 8.4 and 50 mM KCl mixture

containing 2.0 mM MgCl2, 0.25 mM each dNTP and 10 pmol of primers EX5 (SEQ. ID. NO:31) (see Table1) and Vial9 (Boehringer). After an initial 5 min denaturation period at 94 °C, the PCR parameters were 30 sec at 94 °C, 45 sec at 60 °C and 2 min at 72 °C for 35 cycles and a final extension step of 10 min at 72 °C. The approximately 600 bp PCR product was cloned and sequenced.

Exp1 sequence has 1276 bp (SEQ. ID. NO:7) and Exp2 has 1144 bp (SEQ. ID. NO:9). These nucleic acid sequences encode two different Expansin proteins and each sequence corresponds to 100 % of the respective coding region.

The complete nucleotide sequences of Exp1 and Exp2 were sent to NCBI data bank and have shown significant homology with Expansins isolated from other species. The highest homology found at the DNA level using the blastn program for Exp1 was 86% with about 600 base pairs of Fragaria x ananassa Exp1 mRNA clone # AF163812, and for Exp2 90 % with about 800 base pairs of Prunus cerasus expansin2 mRNA clone #AF350937. Searches in all the available protein and DNA data banks failed to find 100 % homology with any existing clone.

The primers used for the first PCR are preferably degenerated primers, which are choosen in conserved portions of different isoforms of the same gene isolated before from other organisms. The other specific primers were designed for 5' and 3' RACE using as template the nucleic acid sequences previously obtained by PCR. Table 1 presents all the designed primers used for gene isolation.

Table1

BG1: 5'-TGG(T/C)TC(T/C)ATTCA(T/C)TA(T/C)CC(T/C)AGAAG-3' (SEQ. ID. NO: 17)

BG2: 5'-CA(C/A/T)GAIC(G/T)(T/A)GGAA(C/T)(A/G)TG(A/G)TACCAT-3' (SEQ. ID. NO:18)

BG3: 5'-GCCTCCATCTTTGGCCTTCTGAAT-3'(SEQ. ID. NO:19)

PG1: 5'-AG(C/T)CC(C/T)AA(C/T)AC(C/T)GA(C/T)GGIAT(C/T)CA-3'(SEQ. ID. NO:20)

PG2: 5'-A(A/G)(A/G)CTICC(A/G)AT(A/G)CT(G/T)ATICC(A/G)TG-3'(SEQ. ID. NO:21)

PG3: 5'-AGTCGAGAATGGTGACTCCAGAT-3'(SEQ. ID. NO:22)

PG4: 5'-GGCACTACCAATTTGTGGATTGA-3'(SEQ. ID. NO:23)

PME1: 5'-ACCGTCGATTTCATTTTCGGA-3'(SEQ. ID. NO:24)

PME2: 5'-AAACCATGGCCTACCAAGATA-3'(SEQ. ID. NO:25)

PME3: 5'-CCCTGTATTGTAATAGTTGCA-3'(SEQ. ID. NO:26)

EX1: 5'-AC(A/G)(A/T)(T/C)GG(T/C)GGITGGTG(T/C)AA(T/C)CC-3'(SEQ. ID. NO:27)

EX2: 5'-TGCCA(G/A)TT(G/T)(G/T)(C/G)ICCCA(A/G)TT(C/T)C-3'(SEQ. ID. NO:28)

EX3: 5'-CGGTATTGGGCAATTTGCAAGAA-3'(SEQ. ID. NO:29)

EX4: 5'-GGATATCGTGAGGGTGAGCGTAA-3'(SEQ. ID. NO:30)

EX5: 5'-GGAGACGTCCATTCAGTTTCAAT-3'(SEQ. ID. NO: 31)

CLAIMS

1. Five isolated nucleic acid sequences from pear fruit comprising encoding regions for β-galactosidase (Pcβ-gal), pectin methylesterase (PcPME), polygalacturonase (PcPG), expansin1 (PcExp1) and expansin2 (PcExp2) proteins.

2. The isolated nucleic acid molecule, according to claim 1, wherein the polynucleotide has the sequence of SEQ. ID. NO:1.

3. The isolated nucleic acid sequence according to claim 2, wherein the polynucleotide encodes a β-Galactosidase polypeptide.

4. The isolated nucleic acid sequences according to claim 2, wherein the polynucleotide encodes a protein or polypeptide having an aminoacid sequence of SEQ. ID. NO:2.

5. The isolated nucleic acid molecule, according to claim 1, wherein the polynucleotide has the sequence of SEQ. ID. NO:3.

6. The isolated nucleic acid sequences according to claim 5, wherein the polynucleotide encodes a Polygalacturonase polypeptide.

7. The isolated nucleic acid sequences according to claim 5, wherein the polynucleotide encodes a protein or polypeptide having an aminoacid sequence of SEQ. ID. NO:4.

8. The isolated nucleic acid molecule, according to claim 1, wherein the polynucleotide has the sequence of SEQ. ID. NO:5.

9. The isolated nucleic acid sequences according to claim 8, wherein the polynucleotide encodes a Pectin methylesterase polypeptide.

10. The isolated nucleic acid sequences according to claim 8, wherein the polynucleotide encodes a protein or polypeptide having an aminoacid sequence of SEQ. ID. NO:6.

11. The isolated nucleic acid molecule, according to claim 1, wherein the polynucleotide has the sequence of SEQ. ID. NO:7.

12. The isolated nucleic acid sequences according to claim 11, wherein the polynucleotide encodes an Expansin polypeptide said Exp1.

13. The isolated nucleic acid sequences according to claim 11, wherein the polynucleotide encodes a protein or polypeptide having an aminoacid sequence of SEQ. ID. NO:8.

14. The isolated nucleic acid molecule, according to claim 1, wherein the polynucleotide has the sequence of SEQ. ID. NO:9.

15. The isolated nucleic acid sequences according to claim 14, wherein the polynucleotide encodes an Expansin polypeptide said Exp2.

16. The isolated nucleic acid sequences according to claim 14, wherein the polynucleotide encodes a protein or polypeptide having an aminoacid sequence of SEQ. ID. NO:10.

17. The isolated nucleic acid sequences according to claim 1, presented as RNA, mRNA, cRNA, DNA or cDNA molecules.

18. A nucleic acid fragment of at least 30 nucleotide homologous to any of the isolated nucleic acid sequences of claim 1.

19. The isolated nucleic acid sequences described in claim 1, which can be used together with other genes expressed in pear fruit.

20. A chimeric gene comprising one or more nucleic acid molecules according to claim 1 in sense or antisense orientation and which can be operably linked to a promoter.

21. A chimeric gene comprising at least one nucleic acid fragment according to claim 18 in sense or antisense orientation and which can be operably linked to a promoter.

22. Any expression cassette comprising at least one of the chimeric genes described in claim 20 and 21.

23. Any replicable expression vector comprising at least one of the chimeric genes described in claim 20 and 21.

24. A plant genome comprising at least one of the chimeric genes described in claim 20 and 21.

25. A host cell transformed with at least one of the chimeric genes described in claim 20 and 21.

26. A genetically modified plant containing at least one of the chimeric genes described in claim 20 and 21, wherein said chimeric gene is stably integrated into the plant genome.

27. The progeny of cross breeding involving the plant described in claim 26.

28. The fruit or seeds comprising at least one of the chimeric genes described in claim 20 and 21, wherein said chimeric gene is stably integrated into the plant genome.

29. Any method of modifying softness in fruits of a plant, the method comprising introduction into the plant an expression cassette according to the described in claim 22.

30. Any method of modifying cell walls in the tissues of a plant, the method comprising introduction into the plant an expression cassette according to the described in claim 22.

31. Any method of modifying plant cell walls response to physiological processes or biological agents, such as fruit ripening or pathogen attack, the method comprising introduction into the plant an expression cassette according to the described in claim 22.

SEQUENCE LISTING

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cctttcaact actcgttcta aagtaattaa gacaagtagc ctctttattt ctcctacatc     60

tccatctcac tccttttttca aatcagaaaa tcctaaaacc agccagcaca a atg gca     117
                                                        Met Ala
                                                        1

aac ccc aaa agc ctc tca tat cca gca gct gca gtt ttt gcg ttg ttg     165
Asn Pro Lys Ser Leu Ser Tyr Pro Ala Ala Ala Val Phe Ala Leu Leu
        5                   10                  15

atg atg gct ata agc att act aat gtg gat gct gca gcc gtc act ttc     213
Met Met Ala Ile Ser Ile Thr Asn Val Asp Ala Ala Ala Val Thr Phe
```

```
    20                  25                  30

agt gtg agc agt tta gga gcc aaa gca gat ggc agt act gac tcc acc      261
Ser Val Ser Ser Leu Gly Ala Lys Ala Asp Gly Ser Thr Asp Ser Thr
35                  40                  45                  50.

aag gcc ttc ctc tct gcg tgg tcc aat gct tgt gcc tcc gtc aac cct      309
Lys Ala Phe Leu Ser Ala Trp Ser Asn Ala Cys Ala Ser Val Asn Pro
                55                  60                  65

gct gtc ata tat gtc ccc gca ggg agg ttc ttg ctt ggc aat gcc gtg      357
Ala Val Ile Tyr Val Pro Ala Gly Arg Phe Leu Leu Gly Asn Ala Val
            70                  75                  80

ttc tct ggg cca tgc aag aac aac gcc atc acc ttc cgc att gcc ggc      405
Phe Ser Gly Pro Cys Lys Asn Asn Ala Ile Thr Phe Arg Ile Ala Gly
        85                  90                  95

act ctc gtc gcc ccg tct gat tac cgg gtc att gga aat gcc ggt aac      453
Thr Leu Val Ala Pro Ser Asp Tyr Arg Val Ile Gly Asn Ala Gly Asn
    100                 105                 110

tgg ctt ctc ttt cag cat gtc aat ggg gtc acg att tcc ggt gga gtt      501
Trp Leu Leu Phe Gln His Val Asn Gly Val Thr Ile Ser Gly Gly Val
115                 120                 125                 130

ctc gac ggt cag ggc acc gga ttg tgg gat tgc aag tcc tcg ggc aag      549
Leu Asp Gly Gln Gly Thr Gly Leu Trp Asp Cys Lys Ser Ser Gly Lys
                135                 140                 145

agt tgc ccc agc gga gca act aca ctg agc ttt tcg aac tcc aac aac      597
Ser Cys Pro Ser Gly Ala Thr Thr Leu Ser Phe Ser Asn Ser Asn Asn
            150                 155                 160

gtt gtg gtg agt gga tta ata tca cta aac agc caa atg ttc cac att      645
Val Val Val Ser Gly Leu Ile Ser Leu Asn Ser Gln Met Phe His Ile
        165                 170                 175

gtc gtc aac ggc tgc caa aat gtg aaa atg caa ggt gtc aag gtt aac      693
Val Val Asn Gly Cys Gln Asn Val Lys Met Gln Gly Val Lys Val Asn
    180                 185                 190

gcg gcc ggc aac agc ccc aac acc gat ggc atc cat gtc caa atg tca      741
Ala Ala Gly Asn Ser Pro Asn Thr Asp Gly Ile His Val Gln Met Ser
195                 200                 205                 210

tct gga gtc acc att ctc gac tcc aaa att tca acc ggt gac gac tgt      789
Ser Gly Val Thr Ile Leu Asp Ser Lys Ile Ser Thr Gly Asp Asp Cys
                215                 220                 225

gtc tca gtt ggc ccc ggc act acc aat ttg tgg att gaa aac gtc gca      837
Val Ser Val Gly Pro Gly Thr Thr Asn Leu Trp Ile Glu Asn Val Ala
            230                 235                 240

tgt gga ccc ggc cac gga atc agc att ggg agt tta ggg aag gac caa      885
Cys Gly Pro Gly His Gly Ile Ser Ile Gly Ser Leu Gly Lys Asp Gln
        245                 250                 255

caa gaa gcc ggt gta caa aat gtt aca gtt aaa aca gtt aca ttc act      933
Gln Glu Ala Gly Val Gln Asn Val Thr Val Lys Thr Val Thr Phe Thr
```

```
    260                 265                 270

ggt act gaa aac ggc gtc aga att aag tct tgg ggg aga cct agc act      981
Gly Thr Glu Asn Gly Val Arg Ile Lys Ser Trp Gly Arg Pro Ser Thr
275                 280                 285                 290

gga ttt gct agg agc att ctt ttc caa cat att gtg atg acc aac gtt     1029
Gly Phe Ala Arg Ser Ile Leu Phe Gln His Ile Val Met Thr Asn Val
                295                 300                 305

caa aat cca atc gtt att gat caa aat tac tgc cct aat gac aaa ggt     1077
Gln Asn Pro Ile Val Ile Asp Gln Asn Tyr Cys Pro Asn Asp Lys Gly
            310                 315                 320

tgc cct ggc caa gct tct gga gtt aag gtc agc gat gtg acg tat caa     1125
Cys Pro Gly Gln Ala Ser Gly Val Lys Val Ser Asp Val Thr Tyr Gln
        325                 330                 335

gac att cat ggt aca tcg gcg acg gaa gtg gcg gtg aaa ttc gat tgt     1173
Asp Ile His Gly Thr Ser Ala Thr Glu Val Ala Val Lys Phe Asp Cys
    340                 345                 350

agt tcc atg tat cct tgc aac ggg atc aga ctg caa gat gtg aag ctc     1221
Ser Ser Met Tyr Pro Cys Asn Gly Ile Arg Leu Gln Asp Val Lys Leu
355                 360                 365                 370

act tac aat aac caa gca gct gaa gct tcc tgc atc cat gca ggc gga     1269
Thr Tyr Asn Asn Gln Ala Ala Glu Ala Ser Cys Ile His Ala Gly Gly
                375                 380                 385

aca act gcc ggt acg gtt cag ccg aca agt tgt ttc taa ctcgagttgt      1318
Thr Thr Ala Gly Thr Val Gln Pro Thr Ser Cys Phe
            390                 395

agttttttcc atctactcct cctcactcgg agtctcgtag tactagttgg gataaaaaag   1378

aagggactag tcatactata aactatatat atatatatat atataagaat taaagaatat   1438

ttctagagta gtaggtctag gtctagctct agctctacgt agttgatgta ttgagatgta   1498

ttttgcttga gcctgccgtg ttggcagcct attgggcttc cttagagcct ggcgctgcat   1558

catccaaacc cacttcatgg agagattctc ttttgcattg ggtgctttgt attatggaat   1618

gttgtaactt gaaagtgata aatgcaatat gaattaaaag taaaaaaaaa aaaaa        1673
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<210> 4
<211> 398
<212> PRT
<213> Pyrus communis
<400> 4

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Met Ala Asn Pro Lys Ser Leu Ser Tyr Pro Ala Ala Ala Val Phe Ala
1               5                   10                  15

Leu Leu Met Met Ala Ile Ser Ile Thr Asn Val Asp Ala Ala Ala Val
            20                  25                  30
```

Thr Phe Ser Val Ser Ser Leu Gly Ala Lys Ala Asp Gly Ser Thr Asp
35 40 45

Ser Thr Lys Ala Phe Leu Ser Ala Trp Ser Asn Ala Cys Ala Ser Val
50 55 60

Asn Pro Ala Val Ile Tyr Val Pro Ala Gly Arg Phe Leu Leu Gly Asn
65 70 75 80

Ala Val Phe Ser Gly Pro Cys Lys Asn Asn Ala Ile Thr Phe Arg Ile
85 90 95

Ala Gly Thr Leu Val Ala Pro Ser Asp Tyr Arg Val Ile Gly Asn Ala
100 105 110

Gly Asn Trp Leu Leu Phe Gln His Val Asn Gly Val Thr Ile Ser Gly
115 120 125

Gly Val Leu Asp Gly Gln Gly Thr Gly Leu Trp Asp Cys Lys Ser Ser
130 135 140

Gly Lys Ser Cys Pro Ser Gly Ala Thr Thr Leu Ser Phe Ser Asn Ser
145 150 155 160

Asn Asn Val Val Val Ser Gly Leu Ile Ser Leu Asn Ser Gln Met Phe
165 170 175

His Ile Val Val Asn Gly Cys Gln Asn Val Lys Met Gln Gly Val Lys
180 185 190

Val Asn Ala Ala Gly Asn Ser Pro Asn Thr Asp Gly Ile His Val Gln
195 200 205

Met Ser Ser Gly Val Thr Ile Leu Asp Ser Lys Ile Ser Thr Gly Asp
210 215 220

Asp Cys Val Ser Val Gly Pro Gly Thr Thr Asn Leu Trp Ile Glu Asn
225 230 235 240

Val Ala Cys Gly Pro Gly His Gly Ile Ser Ile Gly Ser Leu Gly Lys
245 250 255

Asp Gln Gln Glu Ala Gly Val Gln Asn Val Thr Val Lys Thr Val Thr
260 265 270

```
Phe Thr Gly Thr Glu Asn Gly Val Arg Ile Lys Ser Trp Gly Arg Pro
        275                 280                 285

Ser Thr Gly Phe Ala Arg Ser Ile Leu Phe Gln His Ile Val Met Thr
    290                 295                 300

Asn Val Gln Asn Pro Ile Val Ile Asp Gln Asn Tyr Cys Pro Asn Asp
305                 310                 315                 320

Lys Gly Cys Pro Gly Gln Ala Ser Gly Val Lys Val Ser Asp Val Thr
                325                 330                 335

Tyr Gln Asp Ile His Gly Thr Ser Ala Thr Glu Val Ala Val Lys Phe
            340                 345                 350

Asp Cys Ser Ser Met Tyr Pro Cys Asn Gly Ile Arg Leu Gln Asp Val
        355                 360                 365

Lys Leu Thr Tyr Asn Asn Gln Ala Ala Glu Ala Ser Cys Ile His Ala
    370                 375                 380

Gly Gly Thr Thr Ala Gly Thr Val Gln Pro Thr Ser Cys Phe
385                 390                 395
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<210> 5
<211> 700
<212> DNA
<213> Pyrus communis
<220>
<221> CDS
<222> (1)..(588)
<223>

<400> 5

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gaa ttg ggc ccg acg tcg cat gct ccc ggc cgc cat ggc cgc ggg att       48
Glu Leu Gly Pro Thr Ser His Ala Pro Gly Arg His Gly Arg Gly Ile
1               5                   10                  15

gca gtg gtg gca aaa gat gga acg gga aac ttt cag acg gtg aaa gag       96
Ala Val Val Ala Lys Asp Gly Thr Gly Asn Phe Gln Thr Val Lys Glu
            20                  25                  30

gcc atg gat gcg gct gat ggg aaa aaa agg ttt gtg att tac gtg aaa      144
Ala Met Asp Ala Ala Asp Gly Lys Lys Arg Phe Val Ile Tyr Val Lys
        35                  40                  45

gca gga gtt tat aag gag aaa att cac agt aat aaa gac ggg att act      192
Ala Gly Val Tyr Lys Glu Lys Ile His Ser Asn Lys Asp Gly Ile Thr
    50                  55                  60

ttg atc gga gat ggt aaa tat tcc acc atc att gtc ggt gat gat agt      240
```

```
Leu Ile Gly Asp Gly Lys Tyr Ser Thr Ile Ile Val Gly Asp Asp Ser
65                  70                  75                  80

gtt gct gga ggt tcc acc atg cca ggc tct gca act att aca atg aca      288
Val Ala Gly Gly Ser Thr Met Pro Gly Ser Ala Thr Ile Thr Met Thr
                85                  90                  95

ggg gat gga ttc ata gcc cgc gac att ggg ttt cag aac aca gca ggg      336
Gly Asp Gly Phe Ile Ala Arg Asp Ile Gly Phe Gln Asn Thr Ala Gly
            100                 105                 110

cca caa gga gag caa gct tta gct cta aac ata gct tct gat cac tct      384
Pro Gln Gly Glu Gln Ala Leu Ala Leu Asn Ile Ala Ser Asp His Ser
        115                 120                 125

gtt ctt tac agg tgc agc att gcg ggt tac cag gat act ctc tac gca      432
Val Leu Tyr Arg Cys Ser Ile Ala Gly Tyr Gln Asp Thr Leu Tyr Ala
    130                 135                 140

cac gct ctc cgt caa ttc tac aga gaa tgc gac atc tac ggc acc gtc      480
His Ala Leu Arg Gln Phe Tyr Arg Glu Cys Asp Ile Tyr Gly Thr Val
145                 150                 155                 160

gat ttc att ttc gga aac gcc gcc gcg gtt ttc caa aac tgc tac ttg      528
Asp Phe Ile Phe Gly Asn Ala Ala Ala Val Phe Gln Asn Cys Tyr Leu
                165                 170                 175

gtt ctt cgt ctt cct cgg aaa aaa ggc tac aac gtt att cta aaa aac      576
Val Leu Arg Leu Pro Arg Lys Lys Gly Tyr Asn Val Ile Leu Lys Asn
            180                 185                 190

gga aga tcc tga cccgggacag aacactgggt ttctctgttc acaacttgca         628
Gly Arg Ser
        195

gaatcgtacc cagctccgaa ttttctccgg taaaacataa gtaccgaatc gtatcttggt    688

aggccatgga aa                                                       700
```

<210> 6
<211> 195
<212> PRT
<213> Pyrus communis
<400> 6

```
Glu Leu Gly Pro Thr Ser His Ala Pro Gly Arg His Gly Arg Gly Ile
1               5                   10                  15

Ala Val Val Ala Lys Asp Gly Thr Gly Asn Phe Gln Thr Val Lys Glu
            20                  25                  30

Ala Met Asp Ala Ala Asp Gly Lys Lys Arg Phe Val Ile Tyr Val Lys
        35                  40                  45

Ala Gly Val Tyr Lys Glu Lys Ile His Ser Asn Lys Asp Gly Ile Thr
    50                  55                  60
```

```
Leu Ile Gly Asp Gly Lys Tyr Ser Thr Ile Ile Val Gly Asp Asp Ser
65                  70                  75                  80

Val Ala Gly Gly Ser Thr Met Pro Gly Ser Ala Thr Ile Thr Met Thr
                85                  90                  95

Gly Asp Gly Phe Ile Ala Arg Asp Ile Gly Phe Gln Asn Thr Ala Gly
            100                 105                 110

Pro Gln Gly Glu Gln Ala Leu Ala Leu Asn Ile Ala Ser Asp His Ser
        115                 120                 125

Val Leu Tyr Arg Cys Ser Ile Ala Gly Tyr Gln Asp Thr Leu Tyr Ala
    130                 135                 140

His Ala Leu Arg Gln Phe Tyr Arg Glu Cys Asp Ile Tyr Gly Thr Val
145                 150                 155                 160

Asp Phe Ile Phe Gly Asn Ala Ala Ala Val Phe Gln Asn Cys Tyr Leu
                165                 170                 175

Val Leu Arg Leu Pro Arg Lys Lys Gly Tyr Asn Val Ile Leu Lys Asn
            180                 185                 190

Gly Arg Ser
        195
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<210> 7
<211> 1276
<212> DNA
<213> Pyrus communis
<220>
<221> CDS
<222> (65)..(841)
<223>

<400> 7

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caatcctatt catcactctc tctcctctct ctgctttctc actccccttt ctttctctcc     60

ggca atg gcc tcc ctt cgc gtc ctc tac att gct ttc atg ctc tca ctc      109
     Met Ala Ser Leu Arg Val Leu Tyr Ile Ala Phe Met Leu Ser Leu
     1               5                   10                  15

ttc atg gag gcc aac gct aga att cca gga gtt tac act ggt ggc cca      157
Phe Met Glu Ala Asn Ala Arg Ile Pro Gly Val Tyr Thr Gly Gly Pro
                20                  25                  30

tgg gag ggc gcc cac gcc acc ttc tac ggt ggc aac gac gcc tct gga      205
Trp Glu Gly Ala His Ala Thr Phe Tyr Gly Gly Asn Asp Ala Ser Gly
```

```
                35                  40                  45

acc atg ggt ggc gct tgc ggg tac gga aac ctc tac agc caa ggc tac      253
Thr Met Gly Gly Ala Cys Gly Tyr Gly Asn Leu Tyr Ser Gln Gly Tyr
        50                  55                  60

ggc gtg aac acg gcg gca ctg agc act gct ctg ttc aac aat ggc ctt      301
Gly Val Asn Thr Ala Ala Leu Ser Thr Ala Leu Phe Asn Asn Gly Leu
    65                  70                  75

agc tgc ggc gcc tgc ttc gag att aag tgc ggc gac gac ccc agg tgg      349
Ser Cys Gly Ala Cys Phe Glu Ile Lys Cys Gly Asp Asp Pro Arg Trp
80                  85                  90                  95

tgc cac cca ggc aac ccc tcc atc tta gtc acc gcc acc aac ttc tgc      397
Cys His Pro Gly Asn Pro Ser Ile Leu Val Thr Ala Thr Asn Phe Cys
                100                 105                 110

cct cct aac ttc gct cag ccc agc gac gac ggc ggg tgg tgc aac cct      445
Pro Pro Asn Phe Ala Gln Pro Ser Asp Asp Gly Gly Trp Cys Asn Pro
            115                 120                 125

ccc cgc acc cat ttc gac ctc gcc atg ccc atg ttc ctc aag atc gcc      493
Pro Arg Thr His Phe Asp Leu Ala Met Pro Met Phe Leu Lys Ile Ala
        130                 135                 140

gag tac aag gcc ggc atc gtc ccc gtc tct tac cgc cga gtt ccg tgc      541
Glu Tyr Lys Ala Gly Ile Val Pro Val Ser Tyr Arg Arg Val Pro Cys
    145                 150                 155

aga aag caa ggc gga gtg aga ttc aca att aac ggt ttc cgt tac ttc      589
Arg Lys Gln Gly Gly Val Arg Phe Thr Ile Asn Gly Phe Arg Tyr Phe
160                 165                 170                 175

aac ctg gtt ctg atc acc aac gtc gcg ggc gca ggg gat atc gtg agg      637
Asn Leu Val Leu Ile Thr Asn Val Ala Gly Ala Gly Asp Ile Val Arg
                180                 185                 190

gtg agc gta aaa ggc gcg aac act gga tgg atg ccg atg agc cgc aac      685
Val Ser Val Lys Gly Ala Asn Thr Gly Trp Met Pro Met Ser Arg Asn
            195                 200                 205

tgg gga caa aac tgg caa tcc aac gca gac ctg gtg ggc cag acc ctg      733
Trp Gly Gln Asn Trp Gln Ser Asn Ala Asp Leu Val Gly Gln Thr Leu
        210                 215                 220

tcg ttt cga gtc acg ggc agt gac agg cgc aca tcc acc tcc cac aac      781
Ser Phe Arg Val Thr Gly Ser Asp Arg Arg Thr Ser Thr Ser His Asn
    225                 230                 235

gtg gca ccc gct gat tgg cag ttc gga caa act ttc acc ggc aag aat      829
Val Ala Pro Ala Asp Trp Gln Phe Gly Gln Thr Phe Thr Gly Lys Asn
240                 245                 250                 255

ttc cgg gtc taa aattaagaag ggaaaaaaaa gtttatccac tatctttaat          881
Phe Arg Val

tttccttttg ggtttttaac ttttttttta aattatcaaa gtttaatttc ccgccatctg    941
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attttcctta attttcccgg gaaaatttgg aagcggtggg agtataaaag taaaagtatt 1001

agatgatgtg gggttaaaag ttaaaattgg gtggtaagat aggtcgaaaa gcgacttctt 1061

ttgcaagtgt ggtgtgcggc aacttttac ttttggtgct ttttttttta ggtttgagtg 1121

ggaggctggt aaaaatttag gtgatccggc caaatagtgc gtgtaaaagg agttgaagcg 1181

gctgcaaata accaacgtgc agcccgcagc tctacccata tcttttctag aattttatat 1241

gatatatatt tcagttagca aaaaaaaaaa aaaaa 1276

<210> 8
<211> 258
<212> PRT
<213> Pyrus communis
<400> 8

Met Ala Ser Leu Arg Val Leu Tyr Ile Ala Phe Met Leu Ser Leu Phe
1 5 10 15

Met Glu Ala Asn Ala Arg Ile Pro Gly Val Tyr Thr Gly Gly Pro Trp
20 25 30

Glu Gly Ala His Ala Thr Phe Tyr Gly Gly Asn Asp Ala Ser Gly Thr
35 40 45

Met Gly Gly Ala Cys Gly Tyr Gly Asn Leu Tyr Ser Gln Gly Tyr Gly
50 55 60

Val Asn Thr Ala Ala Leu Ser Thr Ala Leu Phe Asn Asn Gly Leu Ser
65 70 75 80

Cys Gly Ala Cys Phe Glu Ile Lys Cys Gly Asp Asp Pro Arg Trp Cys
85 90 95

His Pro Gly Asn Pro Ser Ile Leu Val Thr Ala Thr Asn Phe Cys Pro
100 105 110

Pro Asn Phe Ala Gln Pro Ser Asp Asp Gly Gly Trp Cys Asn Pro Pro
115 120 125

Arg Thr His Phe Asp Leu Ala Met Pro Met Phe Leu Lys Ile Ala Glu
130 135 140

Tyr Lys Ala Gly Ile Val Pro Val Ser Tyr Arg Arg Val Pro Cys Arg
145 150 155 160

Lys Gln Gly Gly Val Arg Phe Thr Ile Asn Gly Phe Arg Tyr Phe Asn

165 170 175

Leu Val Leu Ile Thr Asn Val Ala Gly Ala Gly Asp Ile Val Arg Val
180 185 190

Ser Val Lys Gly Ala Asn Thr Gly Trp Met Pro Met Ser Arg Asn Trp
195 200 205

Gly Gln Asn Trp Gln Ser Asn Ala Asp Leu Val Gly Gln Thr Leu Ser
210 215 220

Phe Arg Val Thr Gly Ser Asp Arg Arg Thr Ser Thr Ser His Asn Val
225 230 235 240

Ala Pro Ala Asp Trp Gln Phe Gly Gln Thr Phe Thr Gly Lys Asn Phe
245 250 255

Arg Val

<210> 9
<211> 1144
<212> DNA
<213> Pyrus communis
<220>
<221> CDS
<222> (83)..(850)
<223>

<400> 9
actccacctg ccctacacaa aaactaaaac tcctctcttt cttttcccta ttgaaatcaa 60

aacccaccaa aaagccacaa aa atg gca gct cat gca ttg tct ttt gct cct 112
Met Ala Ala His Ala Leu Ser Phe Ala Pro
1 5 10

ata gcc ctc tct gtt gtt ctc ttt aat cta cat ctg cat ggt gta ttt 160
Ile Ala Leu Ser Val Val Leu Phe Asn Leu His Leu His Gly Val Phe
15 20 25

gct gtt tat ggt agc tgg gaa ggc gct cat gcc aca ttt tac ggt ggc 208
Ala Val Tyr Gly Ser Trp Glu Gly Ala His Ala Thr Phe Tyr Gly Gly
30 35 40

ggt gat gct tct ggc aca atg gga gga gca tgt ggt tat ggg aat ttg 256
Gly Asp Ala Ser Gly Thr Met Gly Gly Ala Cys Gly Tyr Gly Asn Leu
45 50 55

tac agc cag ggg tat gga acc aac act gca gct ttg agc aca agc att 304
Tyr Ser Gln Gly Tyr Gly Thr Asn Thr Ala Ala Leu Ser Thr Ser Ile
60 65 70

gtt caa caa tgg ctt aag ctg tgg gtc ttg tta tga aat gag atg cga 352

```
Val Gln Gln Trp Leu Lys Leu Trp Val Leu Leu     Asn Glu Met Arg
75                  80                  85

caa tga ccc gag atg gtg ccg tcc tgg atc cat cat tgt aac tgc tac      400
Gln     Pro Glu Met Val Pro Ser Trp Ile His His Cys Asn Cys Tyr
90                  95                  100

aaa ctt ttg ccc tcc taa ctt tgc tca gtc caa cga caa tgg cgg atg      448
Lys Leu Leu Pro Ser     Leu Cys Ser Val Gln Arg Gln Trp Arg Met
105                 110                 115

gtg caa tcc tcc tct cca gca ttt cga ttt ggc tga gcc tgc ttt ctt      496
Val Gln Ser Ser Ser Pro Ala Phe Arg Phe Gly     Ala Cys Phe Leu
120                 125                 130

gca aat tgc cca ata cca gtg ctg gaa tca gtg cca ggt ttc ctt cag      544
Ala Asn Cys Pro Ile Pro Val Leu Glu Ser Val Pro Gly Phe Leu Gln
135                 140                 145                 150

aag agt acc ttg tgt gaa gaa agg agg aat aag att cac cat caa cgg      592
Lys Ser Thr Leu Cys Glu Glu Arg Arg Asn Lys Ile His His Gln Arg
                155                 160                 165

cca ctc cta ctt caa cct ggt ttt gat cac caa cgt ggc tgg ggc agg      640
Pro Leu Leu Leu Gln Pro Gly Phe Asp His Gln Arg Gly Trp Gly Arg
            170                 175                 180

aga cgt cca ttc agt ttc aat caa ggg gtc cag aac agg gtg gca acc      688
Arg Arg Pro Phe Ser Phe Asn Gln Gly Val Gln Asn Arg Val Ala Thr
        185                 190                 195

cat gtc aag aaa ctg ggg tca aaa ctg gca gag caa ctc tta cct caa      736
His Val Lys Lys Leu Gly Ser Lys Leu Ala Glu Gln Leu Leu Pro Gln
    200                 205                 210

tgg cca agc cct ctc ctt cca agt cac cac cag tga cgg tag aac cgt      784
Trp Pro Ser Pro Leu Leu Pro Ser His His Gln     Arg     Asn Arg
215                 220                 225

cac gag cta caa cgt cgc gcc tgg taa ttg gca gtt tgg tca gac att      832
His Glu Leu Gln Arg Arg Ala Trp     Leu Ala Val Trp Ser Asp Ile
    230                 235                 240

ctc cgg ggg tca act tta gagatattcc tctacattat tggtaaaaat            880
Leu Arg Gly Ser Thr Leu
    245

ttgtatatct atctgtcatt tttttcccgt aaacttttt gagtgtaaaa gcaaagagta    940

gttgtgaagt ggaggtttgc tgaggtgagc taaaaaaaca cccgctgggc ctttcacatt   1000

tgagttttcc tggagaaatg atattcacct cattcaggtt gtaaccaatt tctcagttgt   1060

acttgtaacc ttaatgatat atatatttat aaaaaacgag aaagctttat caagtaaaaa   1120

aaaaaaaaag aaaaaaaaaa aaaa                                         1144
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<210> 10

<211> 85
<212> PRT
<213> Pyrus communis
<400> 10

Met Ala Ala His Ala Leu Ser Phe Ala Pro Ile Ala Leu Ser Val Val
1 5 10 15

Leu Phe Asn Leu His Leu His Gly Val Phe Ala Val Tyr Gly Ser Trp
20 25 30

Glu Gly Ala His Ala Thr Phe Tyr Gly Gly Gly Asp Ala Ser Gly Thr
35 40 45

Met Gly Gly Ala Cys Gly Tyr Gly Asn Leu Tyr Ser Gln Gly Tyr Gly
50 55 60

Thr Asn Thr Ala Ala Leu Ser Thr Ser Ile Val Gln Gln Trp Leu Lys
65 70 75 80

Leu Trp Val Leu Leu
85

<210> 11
<211> 5
<212> PRT
<213> Pyrus communis
<400> 11

Asn Glu Met Arg Gln
1 5

<210> 12
<211> 19
<212> PRT
<213> Pyrus communis
<400> 12

Pro Glu Met Val Pro Ser Trp Ile His His Cys Asn Cys Tyr Lys Leu
1 5 10 15

Leu Pro Ser

<210> 13
<211> 21
<212> PRT
<213> Pyrus communis
<400> 13

Leu Cys Ser Val Gln Arg Gln Trp Arg Met Val Gln Ser Ser Ser Pro
1 5 10 15

Ala Phe Arg Phe Gly
20

<210> 14
<211> 95
<212> PRT
<213> Pyrus communis
<400> 14

Ala Cys Phe Leu Ala Asn Cys Pro Ile Pro Val Leu Glu Ser Val Pro
1 5 10 15

Gly Phe Leu Gln Lys Ser Thr Leu Cys Glu Glu Arg Arg Asn Lys Ile
20 25 30

His His Gln Arg Pro Leu Leu Leu Gln Pro Gly Phe Asp His Gln Arg
35 40 45

Gly Trp Gly Arg Arg Arg Pro Phe Ser Phe Asn Gln Gly Val Gln Asn
50 55 60

Arg Val Ala Thr His Val Lys Lys Leu Gly Ser Lys Leu Ala Glu Gln
65 70 75 80

Leu Leu Pro Gln Trp Pro Ser Pro Leu Leu Pro Ser His His Gln
85 90 95

<210> 15
<211> 10
<212> PRT
<213> Pyrus communis
<400> 15

Asn Arg His Glu Leu Gln Arg Arg Ala Trp
1 5 10

<210> 16
<211> 13
<212> PRT
<213> Pyrus communis
<400> 16

Leu Ala Val Trp Ser Asp Ile Leu Arg Gly Ser Thr Leu
1 5 10

<210> 17
<211> 24
<212> DNA
<213> artificial sequence

<220>
<223> Degenerated primer

<400> 17
tggytcyatt caytayccya gaag 24

<210> 18
<211> 24
<212> DNA
<213> artificial sequence
<220>
<223> Degenerated primer

<220>
<221> misc_feature
<222> (1)..(24)
<223> Ionosine

<400> 18
cahganckwg gaayrtgrta ccat 24

<210> 19
<211> 24
<212> DNA
<213> artificial sequence
<220>
<223> designed specific primer

<400> 19
gcctccatct ttggccttct gaat 24

<210> 20
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